Our biobank has a few limitations. First, we enrolled a convenience sample of children depending on the availability of study staff. However, in this study, the proportion of children with Lyme disease did not differ between enrolled and unenrolled but eligible patients. Second, some children with early or early-disseminated Lyme disease might have had false negative serologic results. However, we conducted follow-up to identify children who had initially negative 2-tier Lyme serologic results but tested positive within 30 days of enrollment. Finally, because our network includes only 8 enrollment sites, we were unable to include all regions to which Lyme disease is endemic.

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Interested collaborators should contact Pedi Lyme Net to discuss potential collaborations.

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Transmission Electron Microscopy Confirmation of *Orientia tsutsugamushi* in Human Bile

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Scrub typhus, the third most frequently reported infectious disease in South Korea, causes serious public health problems. In 2019, we collected a bile specimen from a patient with scrub typhus through percutaneous transhepatic gallbladder drainage and performed transmission electron microscopy to confirm the ultrastructure of *Orientia tsutsugamushi*.

Orientia tsutsugamushi is a gram-negative obligately intracellular coccobacillus and a causative pathogen of scrub typhus, which is transmitted to humans by bites from trombiculid (chigger) mites (*1*). Scrub typhus is a prevalent acute febrile disease that mainly occurs in the Asia-Pacific region, infecting ≈1 million persons worldwide each year. In South Korea, infections have increased rapidly since 2014 because of climate change, increased outdoor activities, and numbers of elderly farmers (*2*). The most typical clinical manifestation of scrub typhus is an eschar at the site of the bite (Figure, panel A); symptoms include fever, headache, muscle pain, nausea, and vomiting (*3*). Without proper diagnosis and antimicrobial drug treatment, severe illness with multiple organ system involvement can occur; the death rate is ≈10% (*4*). Immunohistochemical staining for *O. tsutsugamushi* antigens have revealed extensive endothelial cell infection in the heart, lung, kidney, pancreas, skin, and brain (*5*). Bacteria also have been detected in cardiac muscle cells and in macrophages in the liver and spleen (*5*,*6*).

In humans, the liver secretes ≈1 L of bile daily into the intestinal tract. However, little information is available about the presence of gram-negative bacteria in bile (*7*). Pathogenic microorganisms must endure potential impediments, such as variations in pH, low oxygen levels, nutrient limitation, and elevated osmolarity, to survive in this harsh environment (*7*). We collected bile from a patient with scrub typhus in South Korea (Figure, panel B) and visualized the ultrastructure of *O. tsutsugamushi* in the clinical sample using transmission electron microscopy.

In 2019, a 68-year-old woman reported fever, drowsy mental state, abdominal pain, and reduced oral intake. These symptoms had begun 7 days earlier. Her vital signs were blood pressure 100/60 mm Hg and body temperature 38.9°C. Laboratory analysis revealed a leukocyte count 10,350/mL (reference range 4,800–10,800/mL), platelet count 45,000/mL (reference 130,000–450,000/mL), serum creatinine 0.4 mg/dL (reference 0.7–1.7 mg/dL), aspartate aminotransferase 31 IU/L (reference 12–33 IU/L), alanine aminotransferase 41 IU/L (reference 5–35 IU/L), total bilirubin 1.72 mg/dL (reference 0.2–1.2 mg/dL), and C-reactive protein 196.86 mg/L (reference <5 mg/L). Abdominal computed tomography scan detected acute cholecystitis, and percutaneous transhepatic gallbladder drainage was performed. The presence of acute cholecystitis in scrub typhus cases is rare (5 [1.1%] instances of 442 cases) (*8*). *O. tsutsugamushi* can

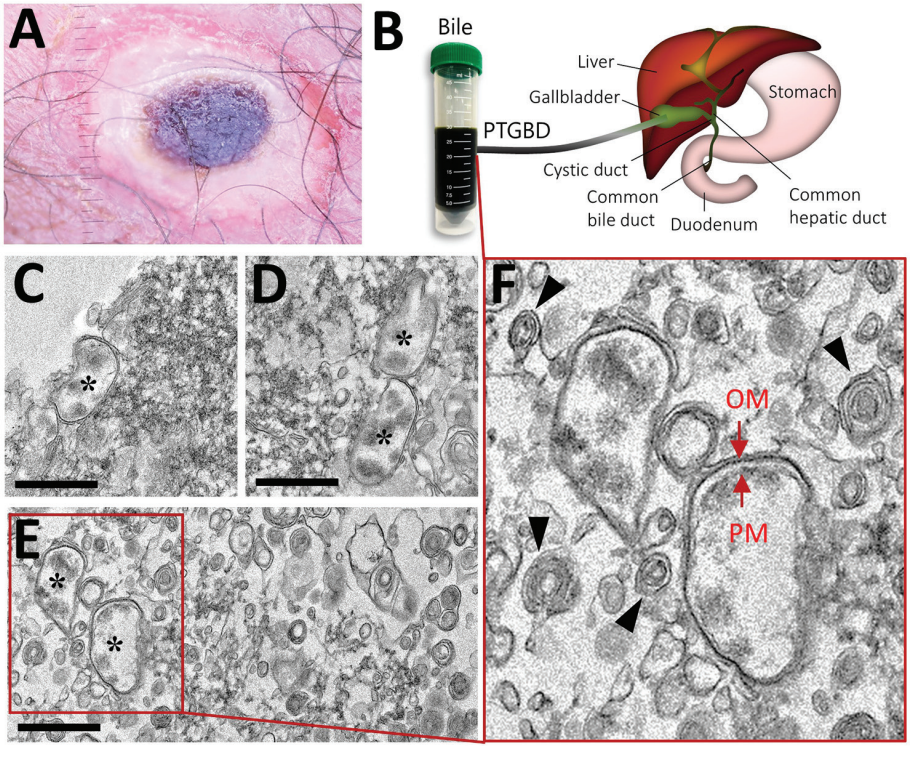

Figure. Findings from a 68-year-old woman with scrub typhus, South Korea, 2019. A) Eschar in the right inguinal area. B) Human bile collected through percutaneous transhepatic gallbladder drainage in the gallbladder of a patient affected with scrub typhus. C–F) Transmission electron microscopy images of *Orientia tsutsugamushi* in the bile. Bacteria (black asterisks); outer membrane (OM) and plasma membrane (PM) (red arrows); multilamellar body (black arrowheads); Scale bars indicate 1 μm.

also cause liver injury in some patients, but the patient reported here did not have any such signs (*9*). We confirmed scrub typhus using indirect immunofluorescence assay (IgG 5,120) and nested PCR selective for the 56-kDa gene of *O. tsutsugamushi* (Appendix, https://wwwnc.cdc.gov/EID/article/26/12/20-2188-App1.pdf). The *O. tsutsugamushi* identified belonged to the Boryong strain (the most common strain in South Korea). We also detected an eschar in the right inguinal area (Figure, panel A). The patient completely recovered after doxycycline treatment.

A drainage tube was placed in the patient's gallbladder, and the bile was directly discharged and collected through the tube (Figure, panel B). We tested the bile specimen for pathogens using nested quantitative reverse transcription PCR and DNA sequencing to detect a specific *O. tsutsugamushi* gene encoding a 56-kDa protein (Appendix) (*10*). After chemical fixation, the sample was embedded in 100% Epon 812 resin and ultrathin (≈80-nm thick) sections were stained with 2% uranyl acetate and 1% lead citrate (Appendix) (*10*). This sample preparation method might not preserve the ultrastructure of live bacteria, but structural features of the bacteria can be clearly observed. The ultrastructural details were acquired using transmission electron microscopy at 120 kV. Despite the presence of a wide variety of components, we detected *O. tsutsugamushi* in the bile (asterisks in Figure, panels C–E). The bacteria showed a coccobacillus shape and were 0.5–0.7-μm in diameter and 1.2–2.5-μm long, all typical features of *O. tsutsugamushi* (*5*,*10*). The bacterial cytoplasm was surrounded by an outer membrane, an internal plasma membrane, and a peptidoglycan layer (Figure, panel F). Moreover, the periplasmic space appeared as an electron-lucent gap between the 2 membranes. We also observed a thicker outer leaflet of the cell wall membrane, which is a typical and diagnostic sign of *Orientia* (*5*). We also detected multilamellar bodies, which are cholesterol-carrying particles, in the bile sample (black arrowheads in Figure, panel F).

Previously, human scrub typhus disease was studied using a mouse model mimicking the disease and examining clinical samples postmortem (*5*,*6*). However, the host cell of *O. tsutsugamushi* in humans has not been completely defined. In this study, we confirmed detection of *O. tsutsugamushi* in human bile, an environment in which bacterial survival is challenging. This observation (i.e., the presence of *O. tsutsugamushi* in human bile) might be useful for diagnosing scrub typhus in patients who do not show clear eschars or skin rash, broadening the potential routes for diagnosing the disease.

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Appendix

Genotyping by DNA Amplification and Sequencing

Peripheral blood mononuclear cells collected from acute-phase blood samples of patients with scrub typhus were purified using a QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Nested PCR was performed. Primers 34 (forward, 5′-TCA AGC TTA TTG CTA GTG CAA TGT CTGC-3′; the 56-kDa gene based on the Gilliam strain) and 55 (5′-AGG GAT CCC TGC TGT GCT TGC TGCG-3′) were used in the first PCR. Nested PCR primers 10 (5′-GAT CAA GCT TCC TCA GCC TAC TAT AAT GCC-3′) and 11 (5′-CTA GGG ATC CCG ACA GAT GCA CTA TTA GGC-3′) were used in the second PCR amplification to generate a 483-bp fragment. Nested PCR was performed as described previously by Kim et al. (*1*). The amplified PCR products were confirmed using 1.2% agarose gel electrophoresis, purified with a QIAquick gel extraction kit (QIAGEN) and sent to COSMO Genetech (Seoul, South Korea) for sequencing.

Identification of 56-kDa Gene by DNA Amplification and Sequencing Using Bile-Derived DNA

Total genomic DNA was extracted from the bile using a QIAamp DNA Mini Kit (QIAGEN), as previously described (*2*,*3*). The 56-kDa gene of *O. tsutsugamushi* was amplified using nested PCR, as previously described (*4*). Briefly, initial rounds of amplifications were performed using touchdown PCR, and products from the initial amplification were used as the template in the second PCR reaction to generate an 83-bp fragment. Nested PCR was performed using Solg 2X Multiplex PCR Smart mix (Solgent, Daejeon, South Korea). Levels of the 56-kDa gene were normalized using the human Gapdh (h*Gapdh*) gene. Information on the primers used in this study is summarized in the Appendix Table. The final PCR products were visualized with a chemiDoc XRS+ system (Bio-Rad,

Hercules, CA, USA) (Appendix Figure 1) and sent to COSMO Genetech for sequencing. The sequencing results were aligned with the original sequence of the 56-kDa gene using Clustal Omega, which revealed the 56-kDa gene in bile-derived DNA (Appendix Figure 2).

Transmission Election Microscopy (TEM)

For ultrastructural analysis of ultrathin sections, collected human bile was pelleted via centrifugation and resuspended in 0.1% glutaraldehyde solution and 4% paraformaldehyde, 3.5% sucrose in phosphate buffer (0.1 M, pH 7.4) for 2 h at 4°C. The samples were washed in phosphate buffer, and then post-fixed in 1% osmium tetroxide (Electron Microscopy Sciences, Hatfield, PA, USA) in phosphate buffer (0.1 M, pH 7.4) for 2 h at 4°C. Samples were dehydrated in a graded ethanol series, followed by propylene oxide, and then progressively infiltrated with a 2:1, 1:1, and 1:2 mixture of propylene oxide and Epon 812 resin (Electron Microscopy Sciences). Samples finally embedded in 100% Epon 812 resin and polymerized at 70°C for 24 h. Ultrathin plastic sections (80-nm thick) were cut at room temperature using a Leica EM UC6 ultramicrotome (Leica Microsystems GmbH, Wetzlar, Germany) and collected on 200-mesh carbon-coated grids. The grids were post-stained with 2% aqueous uranyl acetate and 1% lead citrate at room temperature for 15 and 5 min, respectively. A FEI Tecnai G2 Spirit Twin 120 KV TEM (FEI Company, Hillsboro, OR, USA) was used for TEM analysis (*4*,*5*)

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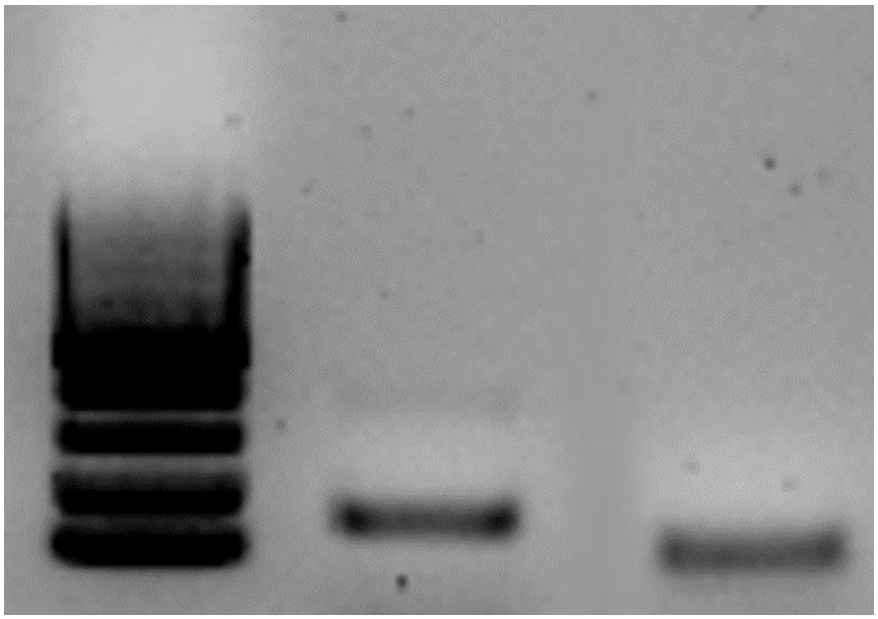
Appendix Table. List of primers used in the study

Primer name	Sequence
c56k F	5′-AGCTGATCGTGACTTTGGGATT-3′
c56k R	5′-AGCATTTGATAATGCAGCAAGACC-3′
rt56k F	5′-CCTAACATACCTCAGGCGCA-3′
rt56k R	5′-AACCAAGCGATCCTAGCTGC-3′
Human GAPDH F	5′-CGGGAAACTGTGGCGTGATG-3′
Human GAPDH R	5′-ATGACCTTGCCCACAGCCTT-3′

*F, forward; R, reverse.



Appendix Figure 1. PCR results in bile-derived DNA of *Orientia tsutsugamushi*. Each lane indicates the ladder and the amplification product obtained using primer pairs for *hGapdh* and *56 kDa*, respectively

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CLUSTAL O(1.2.4) multiple sequence alignment


PCR_product      CCTAACATACCTCAGGCGCAAGCACAAGCGGCGCGGCCTCAGCTAATATGATGAGCACGT   60
56kDa            CCTAACATACCTCAGGCGCAAGCGCAAGCTGCACAGCCTCCGCTTAATGATCAGAAGCGT   60
                 ********************** ***** ** * ***** *** *           ***

PCR_product      GCTGCAGCTAGGATCGCTTGGTTA       84
56kDa            GCTGCAGCTAGGATCGCTTGGTT-       83
                 ************************
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Appendix Figure 2. Sequence alignment for *Orientia tsutsugamushi*.